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Modulation of membrane currents and mechanical activity by niflumic acid in rat vascular smooth muscle

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Abstract

The effects of niflumic acid on whole-cell membrane currents and mechanical activity were examined in the rat portal vein. In freshly dispersed portal vein cells clamped at −60 mV in caesium (Cs^+)-containing solutions, niflumic acid (1–100 μM) inhibited calcium (Ca^{2+})-activated chloride currents ($I_{Cl(Ca)}$) induced by caffeine (10 mM) and by noradrenaline (10 μM). In a potassium (K^+)-containing solution and at a holding potential of −10 mV, niflumic acid (10–100 μM) induced an outward K^+ current ($I_{K(ATP)}$) which was sensitive to glibenclamide (10–30 μM). At concentrations < 30 μM and at a holding potential of −2 mV, niflumic acid had no effect on the magnitude of the caffeine- or noradrenaline-stimulated current ($I_{BK(Ca)}$) carried by the large conductance, Ca^{2+}-sensitive K^+ channel (BK_{Ca}). However, at a concentration of 100 μM, niflumic acid significantly inhibited $I_{BK(Ca)}$ evoked by caffeine (10 mM) but not by NS1619 (1-(2′-hydroxy-5′-trifluoromethylphenyl)-5-trifluoromethyl-2(3*H*)benzimidazolone; 20 μM). In Cs^+-containing solutions, niflumic acid (10–100 μM) did not inhibit voltage-sensitive Ca^{2+} currents. In intact portal veins, niflumic acid (1–300 μM) inhibited spontaneous mechanical activity, an action which was partially antagonised by glibenclamide (1–10 μM), and contractions produced by noradrenaline (10 μM), an effect which was glibenclamide-insensitive. It is concluded that inhibition of $I_{Cl(Ca)}$ and stimulation of $I_{K(ATP)}$ both contribute to the mechano-inhibitory actions of niflumic acid in the rat portal vein.

Keywords: Cl^- current; Niflumic acid; Noradrenaline; Caffeine; Glibenclamide; Smooth muscle

1. Introduction

Numerous patch-clamp studies have demonstrated that noradrenaline stimulates a calcium (Ca^{2+})-sensitive chloride (Cl^-) current ($I_{Cl(Ca)}$) in vascular smooth muscle (Byrne and Large, 1988; Pacaud et al., 1989b; Amédée et al., 1990). As the Cl^--equilibrium potential of smooth muscle is approximately −25 mV (Aickin and Brading, 1982; Aickin and Vermue, 1983), activation of $I_{Cl(Ca)}$ may represent a mechanism by which noradrenaline depolarises the smooth muscle membrane and opens voltage-sensitive Ca^{2+} channels (Amédée and Large, 1989). However, because of the lack of selective inhibitors, the contribution of $I_{Cl(Ca)}$ to the mechanical responses of intact vascular preparations to noradrenaline is unclear.

Niflumic acid is an aromatic, nonsteroidal anti-inflammatory agent (Hoffman and Faure, 1966; Boissier et al., 1967; Ham et al., 1972) and a potent inhibitor of the anion antiporter in erythrocytes (Cousin and Motais, 1979). Niflumic acid also inhibits $I_{Cl(Ca)}$ and volume- and cAMP-activated Cl^- conductances present in various preparations (White and Aylwin, 1990; Korn et al., 1991; Hughes and Segawa, 1993; Martin and Shuttleworth, 1994; Currie et al., 1995). Moreover, niflumic acid attenuates $I_{Cl(Ca)}$ present in both rat and rabbit portal vein (Pacaud et al., 1989a; Hogg et al., 1994b) and in rabbit coronary artery (Lamb et al., 1994). However, quantitative studies of the action of niflumic acid on membrane currents in rat portal vein have not been performed. Furthermore, apart from some preliminary observations (Kirkup et al., 1994; Criddle et al., 1995), few data exist concerning the action of this agent on the mechanical activity of intact vascular preparations.

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The objective of the present investigation was thus to correlate the actions of niflumic acid on membrane currents with those on mechanical activity in the intact vessel. Using this approach, it was hoped to determine the suitability of this agent in clarifying the role of Cl^- channels in vascular smooth muscle excitability.

2. Materials and methods

2.1. Animals

All experiments were performed on portal vein tissue removed from male Sprague-Dawley (Charles River, UK) rats (100–200 g body weight), previously killed by stunning and bleeding. Electrophysiological experiments utilised single, isolated smooth muscle cells while mechanical studies were carried out on whole veins.

2.2. Isolation of cells

Smooth muscle cells were dispersed from whole portal veins as previously described (Ibbotson et al., 1993a; Kirkup et al., 1996) and used within 9 h of separation. All experiments were conducted at room temperature (22–24°C) using patch pipettes made from Pyrex glass (Cat. No. 687-055, Jencons, UK) and of resistance 3–8 MΩ when filled with pipette solution.

2.3. Single-cell electrophysiology

The methods used to obtain electrophysiological responses from single portal vein cells are described extensively elsewhere (Kirkup et al., 1996). Briefly, whole-cell membrane currents were measured in single cells using either the conventional whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) or perforated-patch recording (Horn and Marty, 1988; Rae et al., 1991), with amphotericin B in the pipette solution (300 μg ml^{-1}). Voltage-clamp protocols were delivered and membrane current responses were obtained by means of an Axopatch-1D amplifier (Axon Instruments). This amplifier was connected to an Axon Instruments TL-1 interface and a computer equipped with pClamp 5.5 software (Axon Instruments).

Membrane current data were stored on digital audio tape (DAT) using a Sony DAT recorder and both current and voltage were monitored continuously on a Gould Windograf. Further data acquisition and calculations were performed using the pClamp 5.5, Multistat 1.12 (Biosoft) and Statworks 1.1 (Cricket Software) software. Current values were not corrected for capacitance or leak components. Reversal potential values were determined by subtracting control currents (which included leak and capacitative currents) from values obtained in the presence of niflumic acid. Thus the resulting difference currents represented only the conductance activated by this agent. The reversal potential of the current was determined from the intersection of the appropriate difference current-voltage relationship.

The dispersed cells were superfused (0.7 ml min^{-1}) with a filtered (Millipore 0.22 μm filter) physiological salt solution (PSS). Noradrenaline (10 μM) and caffeine (10 mM) were each applied to the cells by pressure ejection from borosilicate pipettes (World Precision Instruments, UK) of tip diameter 2–4 μm for a period of 400–500 ms or 1–2 s, respectively. The tips of application pipettes were positioned 40–80 μm from the cell surface and pneumatic pressures of 10–14 psi, generated with a PV280 Pneumatic Picopump (WPI, UK), were applied. Electrophysiological responses were only examined in those cells which appeared relaxed, were phase-dense and which contracted in response to application of the agonist in use.

2.4. Mechanical studies

Whole portal veins were quickly excised and mounted under 10 mN tension in 20 ml organ baths containing Krebs-Henseleit solution (KRH solution; composition given below). The solution was maintained at 37°C and gassed continuously with a mixture of 95% O_2 and 5% CO_2 to give a pH of 7.40. Changes in isometric tension were recorded using UF1 Dynamometer strain gauges interfaced to MacLab hardware (MacLab 8, Analog Digital Instruments) and software (Chart, version 2.5).

The preparations were left to equilibrate for 1 h to develop a consistent level of spontaneous activity. During this period they were washed with fresh KRH solution every 15 min and the imposed basal tension was readjusted to 10 mN to compensate for relaxation. When a cumulative protocol for the administration of agents was adopted, the response to each concentration was allowed to develop fully before the next addition was made. A plateau response was usually obtained within 5–10 min for each of the agents used.

2.5. Effects of niflumic acid on spontaneous activity

A cumulative concentration-inhibition curve was constructed for niflumic acid. The tissues were then repeatedly washed with fresh KRH solution until control levels of activity resumed. A subsequent concentration-inhibition curve was then constructed after the tissues had been exposed to either 1 μM or 10 μM glibenclamide for 30 min.

2.6. Effects of niflumic acid on maintained contractions and tension development

Tissues were precontracted with noradrenaline (10 μM). A control concentration-inhibition curve was then made by cumulative addition (0.5 $\log_{10}$ increments) of niflumic

acid in the continuing presence of the agonist. A further concentration-inhibition curve to niflumic acid was subsequently constructed after the preparations had been incubated for 20 min in the presence of 10 μM glibenclamide. In some experiments, tissues were exposed to noradrenaline (10 μM) for a period of 1 min before the agonist was washed off. A 20 min incubation with niflumic acid was then allowed prior to re-exposure to the agonist. The protocol was then repeated after the tissues had been exposed to 10 μM or 30 μM glibenclamide or vehicle for 20 min.

2.7. Statistical analysis

Where applicable, results are expressed as the mean ± S.E.M. with the number of observations in parentheses. Differences between data were assessed using the Student's *t*-test (paired or unpaired as appropriate) in the case of two groups, or one- or two-way analysis of variance in the case of multiple comparisons and were considered to be significant when $P < 0.05$.

2.8. Solutions

2.8.1. Patch-clamp studies

The normal external physiological salt solution (K^+-PSS) was of the following composition (mM): NaCl 126, KCl 6, $MgCl_2$ 1.2, $CaCl_2$ 1.5, HEPES 10, glucose 11. The normal pipette solution (K^+-pipette) comprised (mM): KCl 126, $MgCl_2$ 1.2, HEPES 10, glucose 11, EGTA 1. In some experiments, KCl was isosmotically replaced with CsCl in both the normal external and pipette solutions in order to inhibit K^+ currents (Cs^+-PSS and Cs^+-pipette, respectively). All these solutions were adjusted to pH 7.20 with 3 M NaOH.

The composition of the Ca^{2+}-free external solution (Ca^{2+}-free PSS) was (mM): NaCl 125, KCl 4.8, $MgCl_2$ 3.7, KH_2PO_4 1.2, EGTA 1.0, glucose 11, HEPES 10, buffered with 3 M NaOH to pH 7.20. The Ca^{2+}-free pipette solution contained (mM): NaCl 5, KCl 120, $MgCl_2$ 1.2, K_2HPO_4 1.2, HEPES 10, EGTA 1.2, glucose 11, oxalacetic acid 5, sodium pyruvate 2, sodium succinate 5, buffered to pH 7.20 with 3 M KOH. The estimated free Ca^{2+} concentration of this solution was < 1 nM. For the measurement of Ca^{2+} currents a different caesium-rich pipette solution (Ca^{2+}-free, Cs^+-pipette solution) was used. This was similar to the Ca^{2+}-free pipette solution, except that the KCl and K_2HPO_4 were each replaced by an equimolar concentration of CsCl and the pH of this solution was adjusted to pH 7.20 with CsOH. All superfusing external solutions were continuously bubbled with O_2 and contained 1 μM propranolol to inhibit any β-adrenoceptor-mediated responses.

The enzyme solution for dispersing cells from portal vein comprised (mM): KOH 130, $CaCl_2$ 0.05, taurine 20, pyruvate 5, creatine 5, HEPES 10, collagenase (0.5–1 mg ml^{-1}; Sigma Type VIII), pronase (0.1–0.2 mg ml^{-1}; Calbiochem), fatty acid-free albumin (1 mg ml^{-1}). The pH was titrated to 7.4 with methanesulphonic acid.

2.8.2. Whole-tissue studies

The composition of the KRH solution was as follows (mM): NaCl 118, KCl 4.75, $MgSO_4$ 1.2, KH_2PO_4 1.19, $NaHCO_3$ 25, $CaCl_2$ 2.55 and glucose 11.1. This solution also contained 1 μM propranolol for the studies in which the effects of niflumic acid on noradrenaline-evoked activity were investigated.

2.9. Drugs

Caffeine was dissolved in the appropriate PSS to give the required concentration for filling pressure-ejection pipettes. Stock solutions of glibenclamide and niflumic acid were each made up in ethanol and diluted in the appropriate PSS. Noradrenaline bitartrate was dissolved in 0.1 N HCl before dilution in PSS to give the required concentration for filling pressure-ejection pipettes. Stock solutions of NS1619 (1-(2′-hydroxy-5′-trifluoromethylphenyl)-5-trifluoromethyl-2(3*H*)benzimidazolone; NeuroSearch) and amphotericin B were made up in dimethyl sulphoxide before subsequent dilution in the appropriate solutions. Propranolol (Zeneca) was dissolved in double-distilled water before addition to the PSS. Unless otherwise stated, all drugs and reagents were obtained from Sigma.

3. Results

3.1. Effects of niflumic acid on caffeine- and noradrenaline-evoked $I_{Cl(Ca)}$

When rat portal vein cells were clamped at −60 mV in Cs^+-PSS, application of caffeine (10 mM; Fig. 1a–c) or noradrenaline (10 μM; Fig. 1d) induced $I_{Cl(Ca)}$ (see Kirkup et al., 1994, 1996 for characterisation of this Cl^- current). Niflumic acid produced a concentration-dependent reduction (1 μM: 19 ± 7%; 10 μM: 57 ± 5%; 100 μM: 89 ± 3%, $n = 5–7$) of $I_{Cl(Ca)}$ evoked by caffeine (10 mM; Fig. 1a–c). Niflumic acid (10 μM) also produced a similar effect on noradrenaline (10 μM)-evoked $I_{Cl(Ca)}$ (65 ± 4%, $n = 7$; $P < 0.05$; Fig. 1d). The amplitude of caffeine- and noradrenaline-induced $I_{Cl(Ca)}$ was partially restored after a 5 min washout of niflumic acid (Fig. 1a–d).

3.2. Stimulation of $I_{K(ATP)}$ by niflumic acid

In K^+-PSS at a holding potential of −10 mV, 10 μM niflumic acid induced a non-inactivating outward current which developed after a period of 3–4 min in six of the eight cells tested and which reached a plateau (49 ± 25 pA) after 7 ± 1 min (Fig. 2a). Subsequent exposure to 30

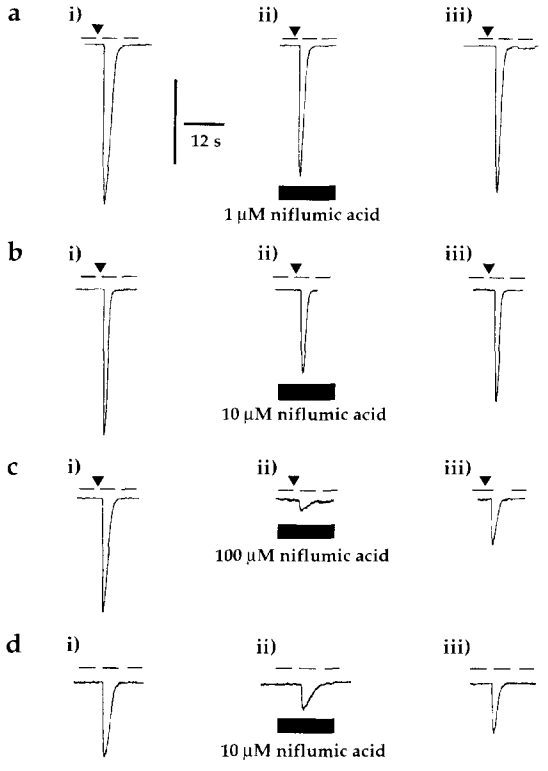

Fig. 1. Effect of niflumic acid on caffeine- and noradrenaline-induced chloride currents ($I_{Cl(Ca)}$) at a holding potential of −60 mV in Cs^+-PSS under perforated patch conditions. (a.i–c.i) $I_{Cl(Ca)}$ was evoked by application of caffeine (▼, 10 mM, 14 psi for 1 s), an effect which was reduced in a concentration-dependent manner after 5 min exposure to niflumic acid 1–100 μM (a.ii–c.ii; bar). The inhibition of $I_{Cl(Ca)}$ was partially reversed after a 5 min washout period (a.iii–c.iii). (d.i) $I_{Cl(Ca)}$ could also be evoked by application of noradrenaline (▽, 10 μM, 10 psi for 500 ms), an effect which was reduced after 5 min exposure to 10 μM niflumic acid (ii, bar). This reduction was partially reversed after a 5 min washout (d.iii). The records (a–d) were obtained from four different cells and the dashed lines denote the zero current. The vertical calibration represents 200 pA in (a), (b) and (c) and 100 pA in (d). The 12 s marker applies throughout.

μM niflumic acid had little additional effect on the magnitude of this outward current (Fig. 2b) but induced a similar current in the two cells which had failed to respond to 10 μM (data not shown). The mean maximum amplitude of the outward current in the presence of 30 μM niflumic acid was 58 ± 19 pA ($n = 8$). Application of 10 μM glibenclamide rapidly reversed these effects of niflumic acid (Fig. 2b).

The properties of this non-inactivating current were investigated using a voltage-step protocol in which cells were held at −10 mV and stepped for 500 ms to a series of test potentials (−80 mV to +20 mV) in 10 mV increments. In the absence of niflumic acid, these voltage steps elicited a family of non-inactivating currents. In the presence of 10 μM and 30 μM niflumic acid, application of the same voltage steps evoked non-inactivating currents of greater amplitude (Figs. 2 and 3). The reversal potential (E_{rev}) of the current induced by 30 μM niflumic acid was −77 ± 2 mV ($n = 6$), a value not significantly different from the calculated K^+-equilibrium potential (−77 mV; $P > 0.05$). Moreover, the zero current potential was shifted from −38 ± 4 mV to −57 ± 4 mV, indicating that the cells had become hyperpolarised (Fig. 3b). In the presence of glibenclamide, these effects of niflumic acid were antagonised (Fig. 3).

3.3. Effects of caffeine- and noradrenaline on K^+ currents in the presence of niflumic acid

At a holding potential of −2 mV, application of caffeine (10 mM; Fig. 4a,b) or noradrenaline (10 μM; Fig. 4c) stimulates $I_{BK(Ca)}$ (Kirkup et al., 1996). In the present study, exposure to niflumic acid (10–30 μM, $n = 3$–5) for 5 min markedly increased $I_{BK(Ca)}$ in two out of 13 cells (see Fig. 4b), but overall this compound had no significant effect on the amplitude of $I_{BK(Ca)}$ induced by caffeine or noradrenaline (niflumic acid 10 μM: +18 ± 12% and

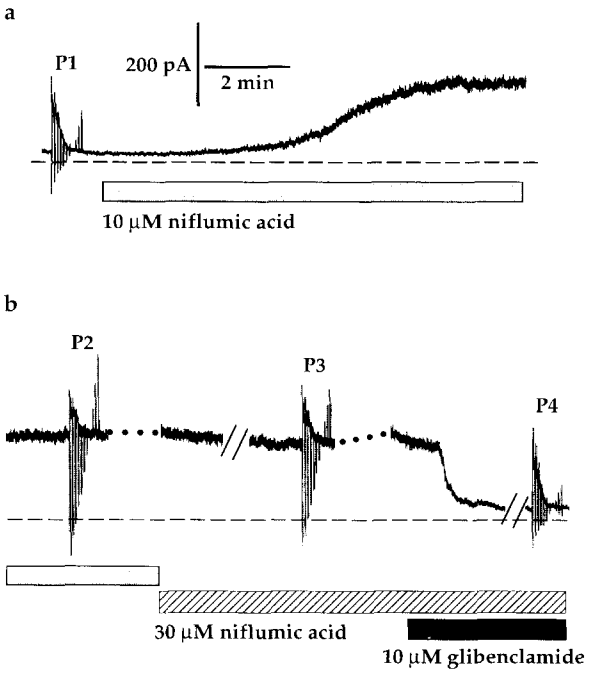

Fig. 2. Effect of niflumic acid on the holding current in a single cell at −10 mV in potassium-containing solutions. (a) Stepping to a series of test potentials ranging from −80 mV to +20 mV in 10 mV increments (P1) generated non-inactivating currents with a reversal potential (E_{rev}) of −30 mV. Exposure to 10 μM niflumic acid (bar) increased the holding current and associated noise, a response which reached a plateau after about 8 min. (b) Re-application of the test potentials (P2) generated larger currents with an E_{rev} of approximately −60 mV. Exposure to 30 μM niflumic acid (bar) had no further effect. Application of glibenclamide (10 μM, bar) rapidly inhibited the niflumic acid-induced increase in holding current and after 5 min in the continued presence of both agents, further application of the clamp protocol (P4) produced a series of currents with an E_{rev} of approximately −30 mV. The dashed line refers to the zero current level. The gaps in the trace (b) marked by diagonal parallel lines were 9 min in length and the dots show in real time where a different step protocol was applied (data not shown).

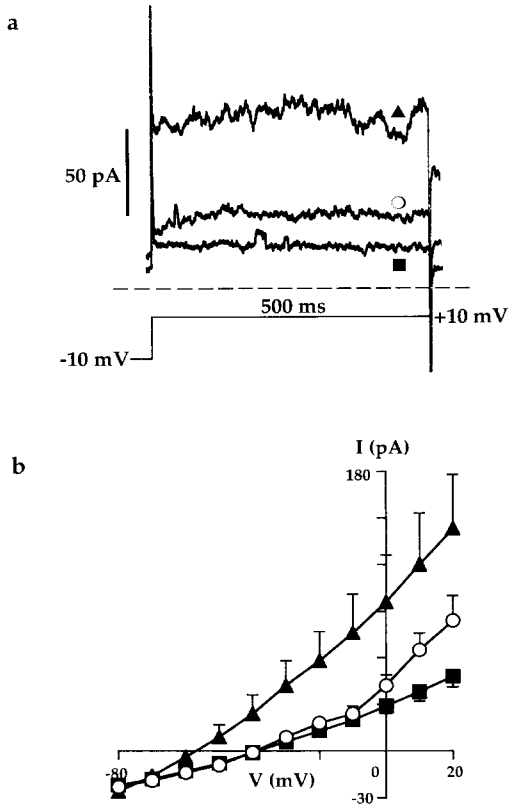

Fig. 3. Induction of $I_{K(ATP)}$ by niflumic acid. Stepping to a test potential of +10 mV from a holding potential of −10 mV elicited a small outward current (○). Exposure to 30 μM niflumic acid (▲) induced a marked increase in the current evoked by the same voltage step, an effect which was reversed after application of 10 μM glibenclamide (■). (b) Current-voltage relationships obtained on stepping to a range of test potentials in 10 mV increments from a holding potential of −10 mV in the (○) absence and (▲) presence of 30 μM niflumic acid and 30 μM niflumic acid plus 10 μM glibenclamide (■). The record (a) was obtained from a single cell and the dashed line denotes the zero current level. Each point in (b) represents the mean ± S.E.M., $n = 6$.

+15 ± 15%, respectively for caffeine- and noradrenaline-induced $I_{BK(Ca)}$, $n = 5$; niflumic acid 30 μM: 32 ± 27% for caffeine-induced $I_{BK(Ca)}$, $n = 3$; see also Fig. 4a and 4c). However, after the induction of $I_{K(ATP)}$ by 100 μM niflumic acid, the magnitude of caffeine-evoked $I_{BK(Ca)}$ in the continuing presence of niflumic acid was greatly reduced (by 72 ± 11%, $n = 8$; $P < 0.005$: Fig. 4a.iii), effects which were not reversed after a 5 min wash (Fig. 4a.iv, $n = 4$). In two of these 8 cells, $I_{BK(Ca)}$ was totally absent. Furthermore, the magnitude of $I_{K(ATP)}$ induced by niflumic acid was also reduced on exposure to caffeine (Fig. 4b.iii). Application of 10 μM glibenclamide reduced the niflumic acid-stimulated increase in $I_{K(ATP)}$ (Fig. 4b.iv; see also Fig. 2). However, neither the inhibition of caffeine-stimulated $I_{BK(Ca)}$ by niflumic acid nor the inhibition of $I_{K(ATP)}$ by caffeine was glibenclamide-sensitive (Fig. 4b.iv, $n = 3$). The vehicle in which niflumic acid was dissolved (0.3% ethanol) had no effect on caffeine-stimulated $I_{BK(Ca)}$ (Fig. 4d).

3.4. Effects of niflumic acid on Ca^{2+} currents in caesium-containing solutions

In caesium-containing solutions, stepping from −60 mV to a series of test potentials (−50 mV to +50 mV in 10 mV increments) stimulated a nifedipine-sensitive (data not shown) calcium current, I_{Ca} (Fig. 5). Exposure to niflumic acid (10 μM to 100 μM) produced no significant effect on either the peak I_{Ca} (control, −165 ± 22 pA; 10

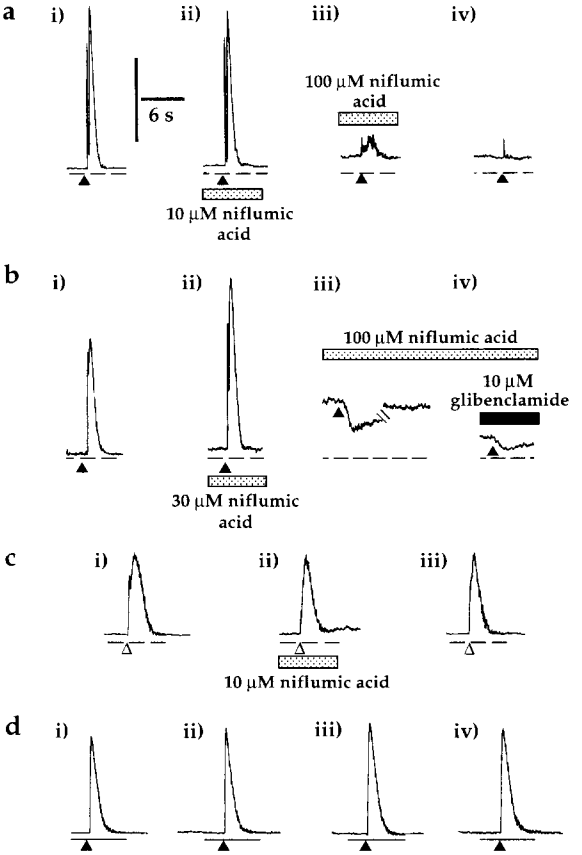

Fig. 4. Effects of niflumic acid on caffeine- (▲, 10 mM, 14 psi for 1 s) and noradrenaline (△, 10 μM, 14 psi for 500 ms) -evoked potassium currents ($I_{BK(Ca)}$) at a holding potential of −2 mV under perforated patch conditions. (a.i and b.i) Application of caffeine elicited $I_{BK(Ca)}$. (a.ii and b.ii) After 5 min exposure (bars) to niflumic acid (10–30 μM), the magnitude of caffeine-activated $I_{BK(Ca)}$ was not attenuated. In contrast, in the presence of 100 μM niflumic acid for 5 min (bar), the induction of $I_{BK(Ca)}$ by caffeine was much reduced (a.iii) and in some cells (a.iv). $I_{K(ATP)}$ induced by niflumic acid (see Fig. 3) was also inhibited (the gap denoted by diagonal parallel lines in trace b.iii is 20 s). These effects were not fully reversed after a 5 min washout (a.iv) or by a 5 min application of 10 μM glibenclamide (b.iv, bar). (c.i–iii) The amplitude of noradrenaline-evoked $I_{BK(Ca)}$ remained constant in the presence of 10 μM niflumic acid and after a 5 min washout of the agent. (d.i–iv) The effects of caffeine applied at 5 min intervals were constant in the presence of the solvent for niflumic acid (0.3% ethanol). The records (a, b, c and d) were obtained from four different cells and the dashed line denotes zero current. The vertical calibration bar represents 400 pA in (a) and (d) and 200 pA in (b) and (c). The horizontal calibration bar applies to all records.

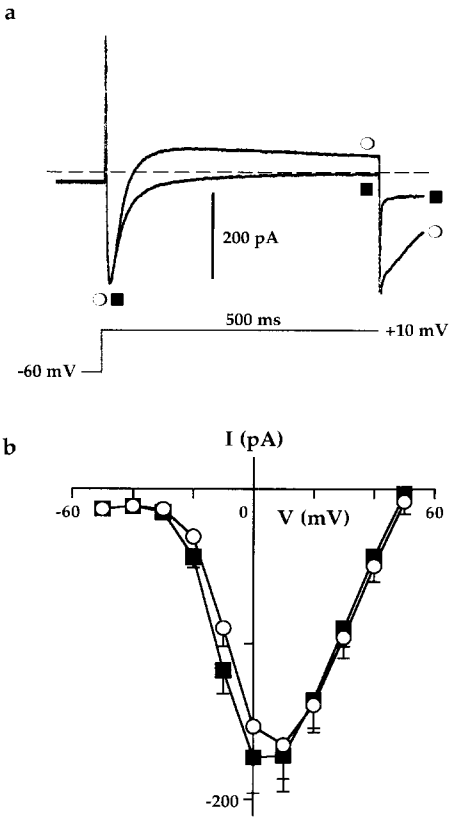

Fig. 5. Effect of niflumic acid on calcium currents (I_{Ca}) in caesium-containing solutions. (a) Stepping to a test potential of +10 mV from a holding potential of −60 mV elicited I_{Ca} (○). The peak inward current was not affected in the presence of 30 μM niflumic acid for 5 min (■). However, an outward current evident at the end of the test pulse and the tail current were markedly reduced in the presence of this agent. The record was derived from a single cell and the horizontal dashed line refers to the zero current level. (b) Current-voltage relationships for peak I_{Ca} evoked on stepping from a holding potential of −60 mV to a series of test potentials for 500 ms in the (○) absence and (■) presence of 30 μM niflumic acid. Each point represents the mean ± S.E.M., $n = 4$–9. Similar results were obtained in the presence of 10 μM and 100 μM niflumic acid but the data have been removed for clarity.

μM, −156 ± 14 pA; 30 μM, −172 ± 23 pA; 100 μM, −165 ± 32 pA, $n = 4$–9) or on the inactivation kinetics of this current (control, 40 ± 3 ms; 10 μM, 39 ± 3 ms; 30 μM, 39 ± 4 ms; 100 μM, 40 ± 3 ms) when generated by the test pulse to +10 mV (Fig. 5). However, in 33% of cells tested both the outward current evident at the end of test pulses and the tail current were inhibited by niflumic acid (Fig. 5a).

3.5. Effects of niflumic acid on $I_{BK(Ca)}$ induced by NS1619 in calcium-free conditions

In essentially Ca^{2+}-free solutions (Ca^{2+}-free pipette solution and Ca^{2+}-free PSS) and using the conventional whole-cell recording configuration, NS1619 induces $I_{BK(Ca)}$ (Edwards et al., 1994). In the present study the induced current reached a plateau within 10 min ($n = 3$, data not shown). After 15 min exposure to 20 μM NS1619, niflumic acid (100 μM for 5 min) had no effect on the increased level of $I_{BK(Ca)}$ (+50 mV; 20 μM NS1619, 1065 ± 225 pA; NS1619 + 100 μM niflumic acid, 1174 ± 315 pA, $n = 3$).

3.6. Effects of niflumic acid on spontaneous activity and on noradrenaline-induced contractions of whole portal veins

Niflumic acid inhibited both spontaneous and noradrenaline (10 μM)-induced mechanical activity of rat portal vein in a concentration-dependent fashion (Fig. 6a,b). The pIC_{50} values for niflumic acid against spontaneous (5.15 ± 0.06, $n = 4$) and evoked activity (4.22 ± 0.11, $n = 4$) were significantly different ($P < 0.01$). The concentration-inhibition curve of niflumic acid on spontaneous activity was displaced to the right in the presence of 1 μM and 10 μM glibenclamide and the pIC_{50} values were altered to 4.30 ± 0.09 ($n = 4$) and 4.40 ± 0.09 ($n = 4$), respectively. These values were both different ($P < 0.005$ and $P < 0.01$, respectively) from the control pIC_{50} but were not different from each other. Conversely, the pIC_{50}

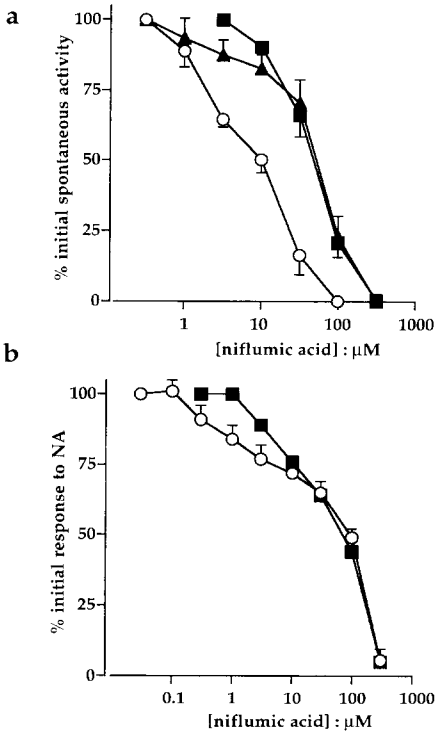

Fig. 6. Mechano-inhibitory effects of niflumic acid in the rat portal vein. (a) Niflumic acid (○) produced a concentration-dependent inhibition of spontaneous activity of the rat portal vein, an action which was antagonised by (▲) 1 μM and (■) 10 μM glibenclamide in a non-competitive fashion. (b) Niflumic acid (○) also produced a concentration-dependent relaxation of tension previously induced by noradrenaline (NA, 10 μM). The spasmolytic effect of niflumic acid on noradrenaline-induced contractions was not antagonised in the presence of 10 μM glibenclamide (■). Each point represents the mean ± S.E.M., $n = 4$–7.

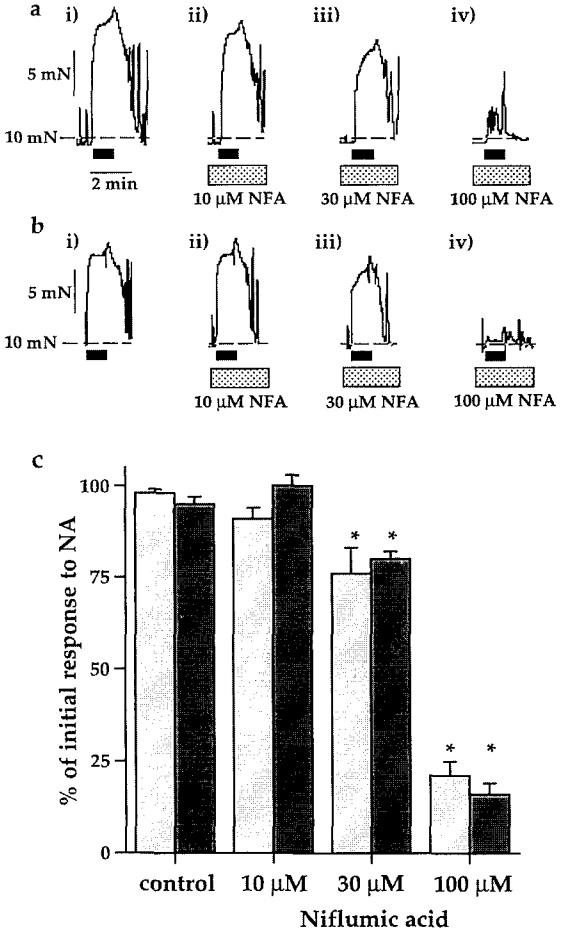

Fig. 7. Effects of niflumic acid (NFA) on noradrenaline (NA)-induced tension development. (a) In the presence of vehicle (0.1% ethanol) or (b) 10 μM glibenclamide, exposure to 10 μM NA for 1 min (bar) evoked a contraction which was attenuated in the presence of 10–100 μM niflumic acid. (c) Mean effects of 10–100 μM niflumic acid on responses to noradrenaline (10 μM, 1 min) as a percentage of the initial contraction, in the presence of vehicle or 10 μM glibenclamide (light and dark columns, respectively). The traces in (a) and (b) were obtained from two tissues and the dashed lines show the 10 mN basal tension marker. The vertical lines on the columns denote the S.E.M., $n = 4$. * Significant difference from the initial response.

value of niflumic acid on noradrenaline-induced activity was not significantly altered (4.25 ± 0.11; $n = 4$) in the presence of 10 μM glibenclamide (Fig. 6b).

3.7. Effect of niflumic acid on maintained noradrenaline-induced contractions

Noradrenaline (10 μM, 1 min) evoked contractions of intact portal veins which were attenuated after subsequent exposure to niflumic acid (10 μM to 100 μM) in a concentration-dependent fashion (Fig. 7a), effects which were not modified by 10 μM glibenclamide (Fig. 7b,c). A higher concentration of glibenclamide, 30 μM, similarly had no effect ($n = 4$, data not shown).

4. Discussion

4.1. Effects of niflumic acid on agonist-evoked $I_{Cl(Ca)}$ and $I_{BK(Ca)}$

In the present study, the potency of niflumic acid ($IC_{50} \sim 10$ μM) against caffeine-evoked $I_{Cl(Ca)}$ in the rat portal vein was similar to that of this agent on $I_{Cl(Ca)}$ in rabbit portal vein (Hogg et al., 1994b). However, in an earlier study in rat portal vein, $I_{Cl(Ca)}$ induced via Ca^{2+} influx (rather than by agonist-stimulated Ca^{2+} release from stores) was more sensitive to inhibition by niflumic acid (Pacaud et al., 1989a). One possible explanation is that two types of calcium-sensitive Cl^- channel (one preferentially activated by Ca^{2+} influx and the other by Ca^{2+} release from stores) with disparate sensitivities to niflumic acid exist in the portal vein.

At the highest concentration tested in the present study, niflumic acid (100 μM) attenuated caffeine-evoked $I_{BK(Ca)}$ and $I_{Cl(Ca)}$. However, this may not have resulted from direct effects on the underlying channels since at this concentration, niflumic acid did not attenuate $I_{BK(Ca)}$ induced by NS1619 under calcium-free conditions. In fact, lower concentrations of niflumic acid (≤ 30 μM) occasionally *enhanced* $I_{BK(Ca)}$ in some cells, an effect characteristic of niflumic acid and other Cl^--channel inhibitors such as DIDS (diisothiocyanostilbene-2,2′-disulphonic acid), SITS (4-acetamido-4′-isothiocyanostilbene-2,2′-sulphonic acid), mefenamic and flufenamic acids in rabbit portal vein (Hogg et al., 1994a,b; Greenwood and Large, 1995).

Although no direct measurements of the intracellular calcium concentration ($[Ca^{2+}]_i$) or of calcium release were made in the present study, the inhibitory effects of niflumic acid on the calcium-sensitive currents $I_{Cl(Ca)}$ and $I_{BK(Ca)}$ in rat portal vein could have been exerted *indirectly* by reducing the amount of stored Ca^{2+} or by inhibiting Ca^{2+} release from an internal store. In support of this view, lower concentrations of niflumic acid, which inhibited the evoked $I_{Cl(Ca)}$, did not inhibit either noradrenaline- or caffeine-induced $I_{BK(Ca)}$. Since $I_{BK(Ca)}$ requires a lower intracellular calcium concentration ($[Ca^{2+}]_i$) for full activation than $I_{Cl(Ca)}$ (Marty et al., 1984; Hogg et al., 1993a,b), a relatively small reduction in $[Ca^{2+}]_i$ by a low concentration of niflumic acid should indeed preferentially attenuate $I_{Cl(Ca)}$.

In their perforated patch study in rabbit portal vein, Hogg et al. (1994b) concluded that niflumic acid directly inhibited $I_{Cl(Ca)}$ by open-channel blockade. This view was essentially based on the action of niflumic acid on the decay time-course of spontaneous transient inward currents and on the voltage dependency of this action. Although this explanation cannot be excluded on the basis of the present study, the possibility that the inhibitory effects of niflumic acid on ion channels are secondary to an action

on Ca^{2+} stores remains a possibility and is the subject of a further investigation.

4.2. Induction of $I_{K(ATP)}$ by niflumic acid

In a recent investigation, exposure of portal vein cells to the Cl^--channel inhibitor NPPB (5-nitro-2-(3-phenylpropylamino)benzoic acid) resulted in the slow development of a non-inactivating, glibenclamide-sensitive K^+ current (Kirkup et al., 1996). Similar observations were made in the present study using niflumic acid. Furthermore, the current-voltage relationship of the induced current exhibited features of $I_{K(ATP)}$ induced by K_{ATP}-channel openers like levcromakalim in rabbit and rat portal vein (Beech and Bolton, 1989; Noack et al., 1992; Ibbotson et al., 1993a,b).

Until recently (Kirkup et al., 1996), the induction of a glibenclamide-sensitive K^+ current by Cl^--channel inhibitors had not been described in smooth muscle. However, one such compound, NPPB, activates K_{ATP} in insulinoma cells (De Weille and Lazdunski, 1990). These workers proposed that NPPB-induced inhibition of anion channels in mitochondrial membranes might produce uncoupling of ATP synthesis, leading to a fall in $[ATP]_i$ and consequent induction of $I_{K(ATP)}$. In fact, because of its protonophoric nature, NPPB may directly uncouple mitochondrial ATP synthesis to produce a depletion of $[ATP]_i$ (Lukacs et al., 1991). Since structural analogues of niflumic acid such as diphenylamine-2-carboxylate and flufenamic acid are known to be mitochondrial uncouplers (Terada and Murakoa, 1972), this mechanism could also explain the induction of $I_{K(ATP)}$ by niflumic acid.

After $I_{K(ATP)}$ had been induced by niflumic acid, the magnitude of this current was sometimes reduced on exposure to caffeine, an action recently reported in pancreatic β-cells (Islam et al., 1995). Although the mechanism underlying this action was not investigated, caffeine also inhibits currents carried by delayed rectifier (K_V) channels (Noack et al., 1990) and thus joins the rapidly expanding list of chemically diverse substances which inhibit both K_V and K_{ATP}. These range from imidazolines like phentolamine (Edwards et al., 1993) to cytochrome P450 inhibitors such as clotrimazole (Edwards et al., 1996) and such findings may indicate structural features common to these two K^+-channel subtypes.

In studies on the actions of niflumic acid in rabbit portal vein, Greenwood and Large (1995) failed to observe the induction of $I_{K(ATP)}$ by this agent. Instead, these workers reported that at concentrations > 50 μM and at a holding potential of 0 mV, niflumic acid evoked a noisy K^+ current. This was not inhibited by glibenclamide but the current was tetraethylammonium-sensitive and outwardly-rectifying. Similar results were obtained with both mefenamic and flufenamic acids and they concluded that the current was $I_{BK(Ca)}$, although confirmatory experiments with a selective inhibitor such as iberiotoxin were not performed. Presumably the ability of niflumic acid to induce $I_{BK(Ca)}$ (and not $I_{K(ATP)}$) in the rabbit portal vein (Greenwood and Large, 1995) yet to stimulate $I_{K(ATP)}$ (and not $I_{BK(Ca)}$) in rat portal vein (present study) reflects species differences.

4.3. Effects of niflumic acid on Ca^{2+} currents

Niflumic acid (10–100 μM) produced no effect on either the magnitude or the inactivation kinetics of I_{Ca}, an observation previously reported in rat portal vein (Pacaud et al., 1989a), mouse pituitary cells (AtT-20 cells; Korn et al., 1991) and in rabbit portal vein (Hogg et al., 1994b). Thus, in contrast to NPPB (Kirkup et al., 1996), niflumic acid exhibits a certain degree of selectivity with respect to this inward divalent cation conductance.

4.4. Inhibition of mechanical activity by niflumic acid: which ion channels are involved?

The profile of actions from single-cell studies suggested that niflumic acid should relax smooth muscle and the effects of this agent on spontaneous and noradrenaline-induced contractions were thus assessed. Spontaneous activity in whole portal veins arises from specialised pacemaker cells present in the vessel wall in which Ca^{2+} influx through voltage-sensitive channels is a crucial step (Jetley and Weston, 1980). Noradrenaline-induced excitation is initiated via α_1-adrenoceptors. The mechanisms which link their activation to contraction in the rat portal vein are complex but probably involve Ca^{2+} release from IP_3-sensitive stores (Loirand et al., 1992; Pacaud et al., 1993; Leprêtre et al., 1994) and influx of Ca^{2+} through voltage-sensitive Ca^{2+} channels (Pacaud et al., 1991; Sayet et al., 1993).

In the present study, spontaneous contractions of rat portal vein were inhibited by niflumic acid, an action which was glibenclamide-sensitive. This suggests that the observed induction of $I_{K(ATP)}$ by this agent in single cells is functionally relevant. However, the antagonism produced by glibenclamide was not competitive, since similar rightward shifts were produced by 1 μM and 10 μM concentrations of this sulphonylurea. Thus at higher concentrations of niflumic acid, other actions contribute to its ability to inhibit spontaneous contractions. From the single-cell studies an inhibitory action of niflumic acid on I_{Ca} can be discounted and it thus seems reasonable to conclude that the inhibition of $I_{Cl(Ca)}$ is responsible. If so, this would strongly suggest a physiological role for Cl_{Ca} in the regulation of spontaneous activity in the rat portal vein.

Niflumic acid also attenuated maintained contractions produced by noradrenaline and in tissues pre-exposed to niflumic acid, contractions to noradrenaline were reduced. These inhibitory effects were not reversed by glibenclamide and thus inhibition of K_{ATP} was almost certainly not involved. It therefore seems that the inhibition of $I_{Cl(Ca)}$ detected in single portal vein cells is the most likely

explanation for the reduction in the contractile effects of noradrenaline in the intact vessel. If this is correct, it highlights the importance of Cl^--channel opening in both the development and maintenance of vascular tone. The relatively non-selective actions of niflumic acid show that its usefulness as a pharmacological tool in smooth muscle is limited. Nevertheless, the data clearly emphasise the therapeutic potential for a *selective* Cl^--channel inhibitor.

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